# Effect of cryopreservation on DNA normality of mice epididymal sperms following *in vitro* preparation with pentoxifylline and *Glycyrrhiza glabra*

**Saad S. Al-Dujaily[1], Muhaned S. Auied[2] ,Abdul Hussein M. Al-Faisal[3]**

1 Department of Clinical Reproductive Physiology, High Institute of Infertility Diagnosis and ART, Al-Nahrain University.

2Department of Applied Embryology, High Institute of Infertility Diagnosis and ART, Al-Nahrain University

3 Department of Genetic Engineering, Institute of Genetic Engineering and Biotechnology, Baghdad University.Corresponding author: aldujaily8@yahoo.com

## Abstract:

### Background:

Many studies conducted on the pentoxifylline (PX) and *Glycyrrhiza glabra (G.glabra)* as motility stimulants showed positive effect on sperm preparation *in vitro* improving the progressive forward movement. However, it was not known the impact of these stimulants on the nature of genetic material, especially after cryopreservation.

### Objective:

The experiment was designed to find out any harmful effect of a medium containing a mixture of PX and aqueous extract of *G.glabra* on the sperm DNA before and after cryopreservation.

### Materials and Methods:

Fifty mature fertile male mice 812- weeks old were used. Epididymal sperms were obtained from caudal region and prepared *in vitro* by direct activation technique using four media namely;PX alone, *G.glabra* alone, a mixture of PX with *G.glabra* and Ham's F-12 (as control medium). Certain sperm function characteristics were examined as well as evaluation of DNA integrity by acridine orange test and comet assay before and after cryopreservation.

### Results:

The results showed a highly significant ($P<0.001$) improvement in the sperm forward movement, with a highly significant ($P<0.001$) decrease in DNA abnormality using acridine orange test and comet assay, following *in vitro* activation by the four media compared to before activation. Following *in vitro* activation and cryopreservation, the results of epididymal sperm showed a highly significant ($P<0.001$) decrease in DNA abnormality by using PX medium alone, *G.glabra* medium alone and the mixture of PX and *G.glabra* compared to control medium and the results before cryopreservation.

### Conclusion:

According to the results of present study, it has been found no toxic effect of medium containing PX and/or *G.glabra* on the epididymal sperms DNA after activation *in vitro* and cryopreservation using acridine orange test and comet assay. The best result obtained when using a medium containing a mixture of PX+ *G.glabra* together. These data could be applied for preparation and activation of epididymal and testicular sperms obtained from obstructive azoospermic men.

**Keyword: Pentoxifylline,** *Glycyrrhiza glabra*, **DNA integrity, cytogenetic tests, Cryopreservation .**

## Introduction:

Assisted reproductive technologies with cryopreservation were widely used to overcome the male infertility in addition to preserve the fertility e.g. before cytotoxic chemotherapy, radiotherapy or certain surgical treatments that may lead to testicular failure or ejaculatory dysfunction(1). Freezing and thawing involve damage to the plasma membrane and acrosome of human spermatozoa as evidenced by significant ultra-structural changes demonstrated by electron microscopy (2) . This damage was exacerbated during the cryopreservation process by a decrease in the lipid content of the membranes, which contain a high proportion of polyunsaturated fatty acids; the decrease in lipid content indicates lipid peroxidation in the presence of reactive oxygen species (3). It was cleared that abnormalities in the male genome characterized by damaged DNA may be indicative of male subfertility regardless of the routine semen parameters (4). Moreover, cryopreservation has been shown to be associated with an increase in the proportion of sperm with DNA fragmentation (5).

In the last years, many studies were concerned with the herbal plants and sperm motility stimulants substances added for culture media *in vitro*. Furthermore, different researches were accomplished using a media containing PX and/or *G.glabra* that resulted in positive effect on active sperm motility. The PX was added to activate sperms and used for Human IUI (6, 7) and *G.glabra* was added to the medium used for mice IVF program (8). However the best *in vitro* sperm preparation and activation results has been observed following mixing the PX and *G.glabra* together.Therefore the aim of this work was to study whether ,there was any harmful effect on the sperm DNA integrity following the preparation and activation of mice epididymal sperm *in vitro* by medium containing PX and/or *G.glabra* before and after cryopreservation technique.

## Materials and Methods:

**Animal management:** Mature fertile mice(Mus musculus) 812- weeks old and 25- 35 gm of weight were obtained from the Animal House at the National Center for Drug Control and Research-Ministry of Health. Room temperature was between 22 -24 °C, the air of the room was changed continuously using ventilating fan. Photoperiod was automatically controlled of (13± 2 hours light from 6 A.M. to 7 P.M. daily regimen).

**-Study Design:** Male mice were sacrificed by cervical dislocation and the epididymis was dissected out. The sperms were obtained from caudal region by flushing method with 1 ml culture medium (Ham's F-12, Sigma- Aldrich Group, Germany) then each sample divided into four media:

**1- Medium (1):** control medium (free-treated with PX and *G.glabra)*.The epididymal sperm activation was done by adding Ham's F-12(0.5 ml).

**2- Medium (2):** The epididymal sperm activation was done by adding (0.5 ml) of *G.glabra* (0.1 mg/ml ,ratio20%).

**3- Medium (3):** The epididymal sperm activation was done by adding (0.5 ml) of PX (0.1 mg/ml).

**4- Medium (4):** The epididymal sperm activation was done by adding (0.5 ml) of PX(0,1mg/ml) and *G.glabra* (0.1 mg/ml, ratio 20%).

All the suspensions were kept in the incubator for 30 minutes at 37°C. After incubation, 0.5 ml of the prepared sperms were taken and placed in cryovial containing cryoprotectant.

**-Epididymal sperm cryopreservation:** The cryopreservation method used was according to Frank (9). Each prepared epididymal sperm was mixed with cryoprotectant (prepared by adding glycerol to the Ham's F-12 and then filtered by using filter Millipore 0.22 µ and exposed to the UV light for sterilization) and placed in cryovial (Nunc Tm, Denmark, capacity 1.8 ml). Each cryovial was hold by cryovial holder (Nunc Tm, Denmark) and left at room temperature for 10 minutes. The samples were hanged in liquid nitrogen vapor for 30 minutes, then embedded in the liquid nitrogen.

**-Thawing:** After one month of cryopreservation, the thawing of sample was done through rapidly transferring cryovial from liquid nitrogen to the water bath (Kotterman,) at 37°C until melting ice, for at least 5 minutes. The sample was mixed with 1:1 ratio culture medium (Ham's F-12) and then incubated for 10 minutes. Then certain sperm functions parameters and DNA integrity test were examined. The thawing process was done according to Frank (9) .

**-Acridine orange protocol:** This test was done according to Tejada (10).

**-Comet Assay Protocol:** A detailed elaboration of the alkaline comet assay procedure used to quantify DNA damage was accomplished as described by Singh (11).

**Ethics:** This study was approved by the ethics committee. All animals received humane care in accordance with the Institution>s guideline and criteria for humane care as outlined by the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

**Statistical analysis:**The data analysis was performed by using SPSS (Statistical Package of Social Science; version 17.0 LED Technology, USA) and Microsoft Excel Work Sheet 2007). Paired sample t- test was applied by SPSS statistical program to compare between the results of cryopreserved and control groups. The differences between the values were considered statistically significant at P value 0.05(12).

## Results:

**-Effect of *in vitro* sperm activation on certain sperm function parameters before and after activation and cryopreservation:**

There was a highly significant (P<0.001) improvement in active sperm motility grades A, B and A+B of sperms treated with PX, Gg and PX+ *G.glabra* media compared with before activation and control Ham's F-12 medium. A highly significant (P<0.001) decrease in active sperm

motility grades A, B and A+B after cryopreservation was observed with PX, *G.glabra* and PX+ *G.glabra* media compared with the control Ham's F-12 medium before and after cryopreservation. There was a highly significant (P<0.001) decrease in morphologically abnormal sperms (MAS) after activation with (PX, *G.glabra* and PX+ *G.glabra* media) than that of before activation and control medium as shown in figure (1).

**- Results of DNA integrity tests:**

**1. Results of Acridine Orange:**

The results of acridine orange were shown in image -1 and figure (2), A highly significant (P<0.001) decline was observed in DNA denaturation after activation and after cryopreservation in all treated media that used for epididymal sperm activation (PX, *G.glabra* and PX with *G.glabra*) compare to before activation. However, by using Ham's F-12 medium, the DNA denaturation of epididymal sperm activation *in vitro* demonstrates a significant (P<0.05) reduction after activation and cryopreservation of treated media. After cryopreservation DNA denaturation showed a significant (P<0.05) increase in control Ham's F-12 medium as compared to that after activation (Figure 2).

**-Results of comet assay:**

In general, intact cells under fluorescent microscope appear round (all DNA incorporated in head of comet, image 2 A), while cells with DNA damage have a tail (comet) that illustrated in (image 2 B), the length of tail of comet was directly related to the amount of DNA damage in the cells.

The results of comet assay after activation and cryopreservation with (PX, *G.glabra* and PX + *G.glabra* media) revealed a highly significant (P<0.001) decrease in comet length, tail length, DNA in tail and tail moment in treated media compared with the before activation and control Ham's F-12 medium before and after cryopreservation. Also the results show a highly significant (P<0.001) increase in DNA of sperm head in treated media compared with the before activation and control Ham's F-12 medium before

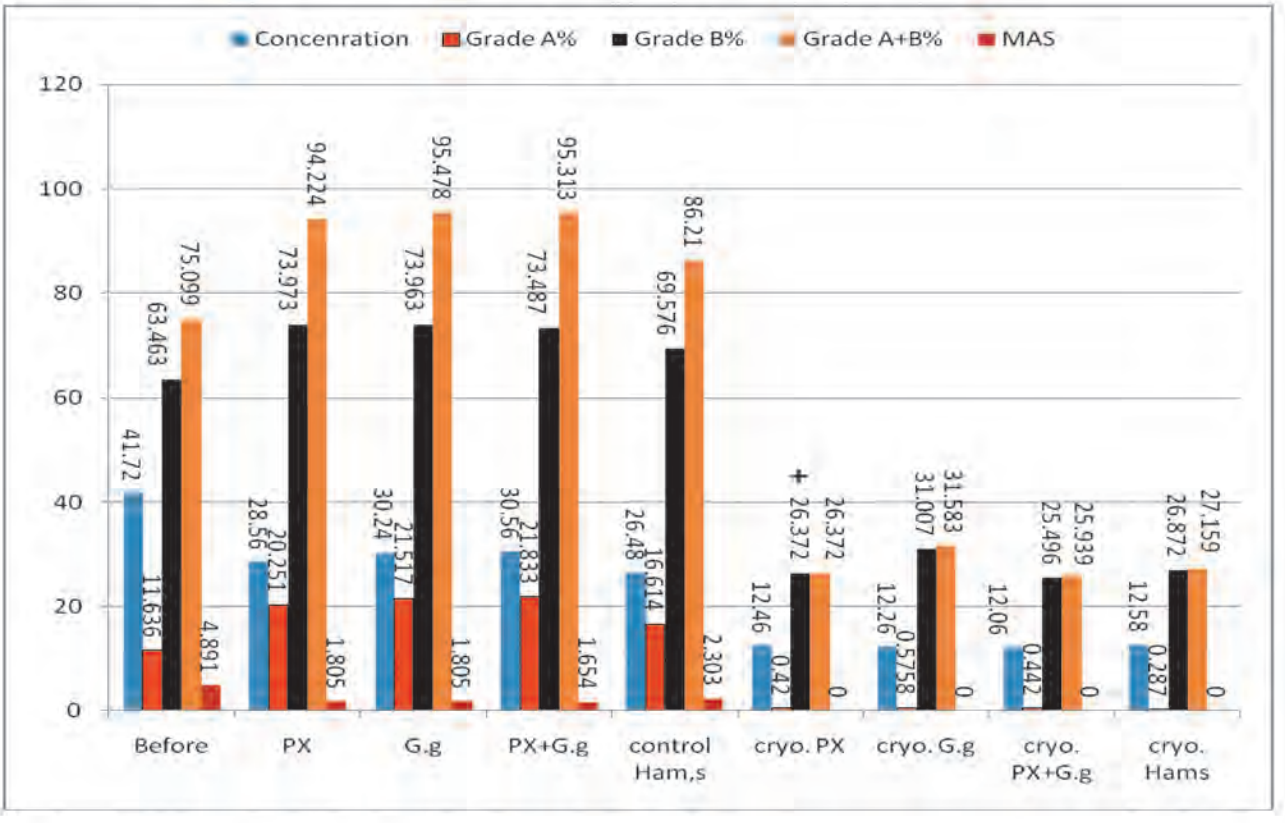


**Figure (1): Histograms showing certain sperm function parameters after *in vitro* sperm activation and cryopreservation with PX, *G.glabra* , PX + *G.glabra* and Hams F-12 media.**

* Highly significant (P<0.001) decrease in sperm concentration in before and after activation and cryopreservation.

** Highly significant (P<0.001) increase in active grade motility between before and after activation.

+ Highly significant (P<0.001) decrease in active grade motility between before and after cryopreservation.

MAS: Morphologically Abnormal Sperm. PX: Pentoxifylline *G.glabra : Glycyrrhiza glabra*

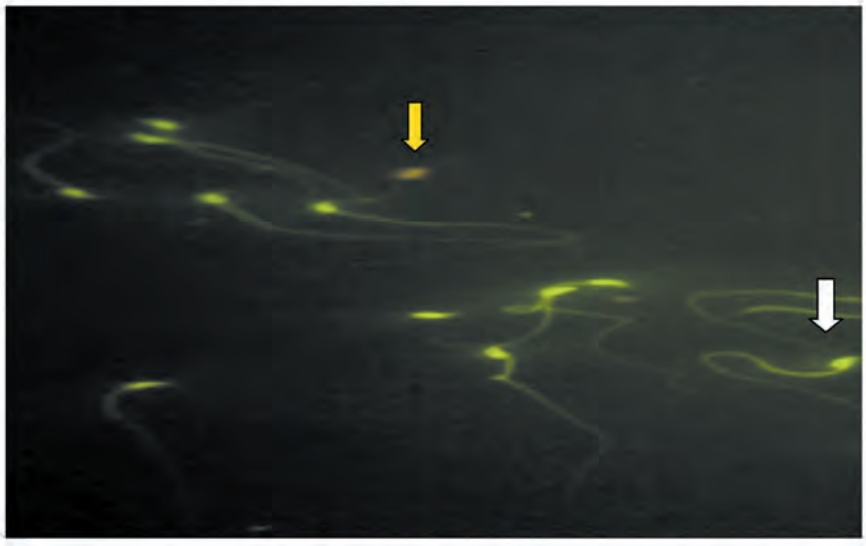

Image (1): Acridine orange test showing sperm normal DNA (white arrow) and sperm DNA denaturation (yellow arrow)

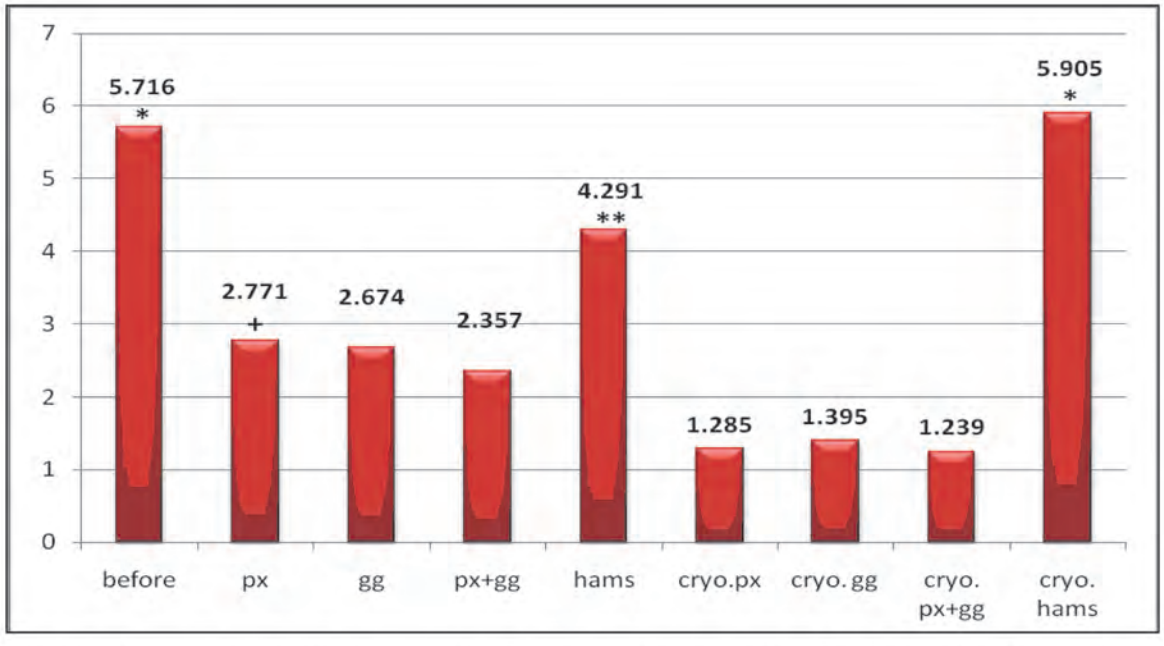


**Figure (2): Histograms showing the percentage of DNA denaturation after activation and cryopreservation on epididymal sperms.**

*P<0.001 a highly significant difference in before and after activation and cryopreservation and then to the control Ham's F-12 medium cryopreservation.

**P<0.05 a significant difference in treated PX medium and control Ham's F-12 medium.+P>0.05 no significant difference in treated PX, PX + *G.glabra* and *G.glabra*..

PX: Pentoxifylline *G.glabra: Glycyrrhiza glabra*

Image (2): SCGE stained with EtBr cell that have intact DNA (white arrow) normal and cell that have DNA damage form a comet (yellow arrow) abnormal.

Table (1): Comet assay results after and before *in vitro* activation and cryopreservation with PX and Ham's F-12 media.

| Parameters | Before activation | After activation | | After cryopreservation | |
|---|---|---|---|---|---|
| | | Treated PX | control Ham's | Treated PX | control Ham's |
| Comet length | 1.909±0.199 | 1.541±0.082* | 1.600±0.933 * | 1.040±0.782 * | 1.938±0.125 |
| Head diameter | 1.342±0.500 | 1.287±0.450 | 1.248±0.318 | 6.464±0.336 | 1.385±.080 |
| DNA in head | 90.912±0.599 | 95.848±0.419* | 94.478±0.428* | 96.904±0.267* | 90.887±0.597 |
| Tail length | 56.689±4.539 | 25.317±3.499* | 35.209±3.233* | 9.376±0.882* | 55.293±4.143 |
| DNA in tail | 9.088±0.599 | 4.152±0.418* | 5.521±0.428* | 3.096±0.266* | 9.113±0.596 |
| Tail moment | 1.501±0.467 | 6.779±1.836 | 6.899±1.636 | 1.159±0.238 | 1.179±0.211 |

**Values are expressed as mean ±SEM. ANOVA**
*Highly significant (P<0.001) differences between before and after activation and cryopreservation.

Table (2): Comet assay results before and after *in vitro* activation and cryopreservation with *G.glabra* and Ham's F-12 media.

| Parameters | Before activation | After activation | | After cryopreservation | |
|---|---|---|---|---|---|
| | | Treated *G.glabra* | Control Ham's | Treated *G.glabra* | control Ham's |
| Comet length | 1.909±0.199 | 1.527±0.635* | 1.600±0.933* | 1.300±0.937* | 1.938±0.125 |
| Head diameter | 1.342±0.500 | 1.401±0.537+ | 1.248±0.318* | 1.122±0.494 | 1.385±.080 |
| DNA in head | 90.912±0.599 | 97.613±0.179* | 94.478±0.428* | 94.538±0.367 | 90.887±0.597 |
| Tail length | 56.689±4.539 | 12.589±0.714* | 35.209±3.233* | 17.80±1.067* | 55.293±4.143 |
| DNA in tail | 9.088±0.599 | 2.387±0.179* | 5.521±0.428* | 5.462±0.367 | 9.113±0.596 |
| Tail moment | 1.501±0.467 | 0.677±0.074* | 6.899±1.636* | 1.997±0.227* | 1.179±0.211 |

**Values are expressed as mean ±SEM, ANOVA**
*Highly significant (P<0.001) differences between before and after activation and cryopreservation.
*Significant (P<0.05) differences between before and after activation.
*G.glabra*: *Glycyrrhiza glabra*

Table (3): Comet assay results after and before *in vitro* activation and cryopreservation with PX + *G.glabra* and Ham's F-12 media.

| Parameters | Before activation | After activation | | After cryopreservation | |
|---|---|---|---|---|---|
| | | Treated PX+ *G.glabra* | control Ham's | Treated PX + *G.glabra* | control Ham's |
| Comet length | 1.909±0.199 | 1.499±0.591* | 1.600±0.933* | 1.234±0.509* | 1.938±0.125 |
| Head diameter | 1.342±0.500 | 1.363±0.377* | 1.248±0.318* | 1.112±0.248* | 1.385±.080 |
| DNA in head | 90.912±0.599 | 97.640±0.160* | 94.478±0.428* | 97.253±0.182* | 90.887±0.597 |
| Tail length | 56.689±4.539 | 13.596±0.749* | 35.209±3.233* | 12.119±0.742* | 55.293±4.143 |
| DNA in tail | 9.088±0.599 | 2.359±0.161* | 5.521±0.428* | 2.746±0.181* | 9.113±0.596 |
| Tail moment | 1.501±0.467 | 0.686±0.079* | 6.899±1.636* | 0.773±0.108* | 1.179±0.211 |

**Values are expressed as mean ±SEM ,ANOVA**

## Discussion

Epididymal sperm activation *in vitro* with the PX, *G.glabra* and PX+ *G.glabra* media noticed positive effect on certain sperm function parameters and DNA normality after activation and cryopreservation by using comet assay and acridine orange stain tests. The action of PX is mediated by inhibition of phosphodiesterase enzyme leading to an increase in cAMP and the *G.glabra* contains Ca++ with different compounds will stimulate the motility of sperm (13) (14). The decrease in the percentage of MAS may resulted from direct activation technique, in addition to the positive effect of both PX and *G.glabra* in stimulating the swim up only of sperm with normal form to move upward. These results are in agreement with those obtained by Al-Dujaily and Malik (15). Moreover, the decrease in DNA abnormality following cryopreservation can be explained by the interaction of PX with the lipid content of the sperm cell membrane, which contains a high proportion of polyunsaturated fatty acids. The positive interaction of PX with polyunsaturated fatty acid may decrease the production of lipid peroxidation which is the source of reactive oxygen species ( ROS) (3).

On the other hand, *G.glabra* contains different antioxidants with different cryoprotectants such as glucose, sucrose, fructose, and maltose. All will exert a positive effect on DNA following cryopreservation of epididymal sperm. However, there was a decline in sperm motility by using the four media following cryopreservation. This finding can be observed because of the nature of epididymal sperm that cannot be equivalent to ejaculate sperm. The caudal region is responsible for protection, maturation and transportation of new product of sperms (17). The mature sperm may resist the external environment whereas the epididymal sperm may contain cytoplasmic droplet which is the source of ROS (18).

The cryopreservation process of epididymal sperms is different from ejaculate sperm because of the increased sensitivity of the latter to DNA damage resulted from enhanced nuclease activity present in the ejaculate sperm of mouse (19). Also Membrane properties between epididymal and ejaculated sperms are different, which may affect sperm survival after cooling and freezing (20). The other explanation that may affect positively in decreasing DNA abnormality is that several dilutions occurs during activation and cryopreservation lead to decrease in abnormal cells and absence of leukocytes may generate ROS. This finding is compatible with other studies that found spermatozoa (21) (22) and leukocytes (23) (24)are the potential sources of ROS .

In addition to PX activation in the mixture medium the *G.glabra* constituents contributed as antioxidants and hepatoprotective properties. Glycyrrhizin and glabridin inhibit the generation of ROS by neutrophils at the site of inflammation (25) (26). *In vitro* studies have demonstrated that licorice contains isoflavones, hispaglabridin A and B, inhibit Fe3+ to induce lipid peroxidation in rat liver cells (27). Other researches indicated that glycyrrhizin lowers lipid peroxide values in animal models of liver injury caused by ischemia reperfusion (28).

Finally, according to the properties of PX, *G.glabra* and glycerol after activation and cryopreservation-thawing process, there was a best result in active sperm motility and DNA integrity which may indicate that PX and *G.glabra* can be used for activation as well as cryoprotectant with the glycerol. All media used for *in vitro* sperm activation (PX, *G.glabra* and PX + *G.glabra* media) kept sperms from freezing damage and did not have any toxic effect on the DNA .

## References


1. Sanger W G, Olson J H and Sherman J K. Semen cryobanking for men with cancer – criteria change. Fertil. Steril. 1992; 58:10241027-.
2. Wooly L and Richardson D W. Ultrastructural injury to human spermatozoa after freezing and thawing. J Reprod. Fertil. 1987; 53:389-394.
3. Cerolini S, Maldjian A and Pizzi F, *et al.* Changes in sperm quality and lipid composition during cryopreservation of boar semen. Reproduction. 2001; 121 395- 401.
4. Aitken R and Krausz C. Oxidative stress, DNA damage and the Y chromosome. Reproduction. 2001; 122: 497 -506.
5. Irvine D, Twigg J and Gordon E *et al.* DNA integrity in human spermatozoa: relationships with semen quality. J Androl. 2000; 21: 33-44.
6. Akerele O. Summary of WHO guidelines for the assessment of herbal medicine, Herbalgram. 1993; 28, 13 -18.
7. Al-Dujaily SS, AL-Janabi A S and Nori M. Effect of Glycyrrhiza extract on *in vitro* sperm activation of asthenospermic patients. J Babylon Uni. 2006; 11(3): 477- 483.
8. Al-Dujaily SS and Al-Saadi A S. Effect of *Glycyrrhiza glabra* extract on IVF outcome in mice. Experimental model for mammals. J. Biotechnol. Res. Center. 2009;3:80 -89.
9. Frank PS. Cryopreservation manual: Nalge Nunc International Corp. 1998.
10. Tejada RI, Mitchell J C and Norman A *et al.* A test for the Practical Evaluation of Male Fertility by Acridine Orange Fluorescence. Fertil. Steril. 1984;42:87 -91.
11. Singh N, McCoy M and Tice R *et al.* A simple technique for quantification of low levels of DNA damage in individual cells. Exp Cell Res 1988; 175: 184–191.
12. Daniel WW. Multiple regression correlation .In: Biostatistics. A foundation for analysis in the health science.1988.
13. Sikka S C and Hellstrom W J. The application of pentoxifylline in the stimulation of sperm motion in men undergoing electroejaculation. J. Androl. 1991; 12,165 -170.
14. Al-Dujaily SS and Al-ani I A. Artificial insemination outcome following washing the cervix and uterus with pentoxifylline. Middle East Fertility Society (MEFs) 4th Annual Scientific Congress Egypt-Cairo, Nov. 2009.
15. Al-Dujaily SS and Malik K. *In vitro* sperm preparation by progesterone, pentoxifylline and *Glycyrrhiza glabra* for asthenozoospermic men. Global JMR (USA).2013; 3(1):16 -2 0-
16. Jones R. To store or mature spermatozoa? The primary role of the epididymis. Int J Androl. 1999; 22 (2): 57–67.
17. Yamauchi Y, Ajduk A and Jonathan M R *et al.* Ejaculated and epididymal mouse spermatozoa are different in their susceptibility to nuclease-dependent DNA damage and in their nuclease activity. Biol. Reprod. 2007; 77, 636–647.
18. Rath D and Niemann H. *In vitro* fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen-thawed epididymal semen obtained from identical boars. Theriogenology. 1997;47:785793-.

19. Bailey J L, Tardif S and Dubé C. The nature of sperm capacitation: current concepts after 50 years of research. In: B. Robaire, H. Chemes and C. Morales (eds.) Andrology in the 21st Century: Proceeding of the VII th International Congress of Andrology: Plenaries, Symposia and Workshops. Medimond Publ., Englewood, NJ, USA. 2001:237 -246.
20. Novotny J, Oborna I and Brezinova J *et al*. The occurrence of reactive oxygen species in the semen of males from infertile couples. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2003;147:173176-.
21. Baker M A, Krutskikh A and Aitken R J. Biochemical entities involved in reactive oxygen species generation by human spermatozoa. Protoplasma. 2003; 221:145- 151.
22. Essayan D M. «Cyclic nucleotide phosphodiesterase. J Allergy Clin. Immunol. 2001; 108 (5): 671–680.
23. Al-Dujaily SS. Detecting Leukocytospermia by Myeloperoxidase staining method for patients of Baghdad IVF Institute. Med J. Babylon. 2005;2(2):200- 204.
24. Akamatsu H, Komura J and Asada Y et al. Mechanism of anti-inflammatory action of glycyrrhizin: effect on neutrophils functions including reactive oxygen species generation. Planta Med. 1991;57:119-121.
25. Wang Z Y and D Nixon W. Licorice and cancer. Nutr. Cancer. 2001;39:111- 115.
26. Haraguchi H, Yoshida N and Ishikawa H et al. Protection of mitochondrial functions against oxidative stresses by isoflavans from *Glycyrrhiza glabra*. J Pharm Pharmacol .2000;52:219- 223.
27. Nagai T, Egashira T and Yamanaka Y et al. The protective effect of glycyrrhizin against injury of the liver caused by ischemia-reperfusion. Arch Environ Contam Toxicol 1991;20:432 -436.